Identification and characterization of acyclovir-resistant clinical HSV-1 isolates from children

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A S T R A C T

Background: The occurrence of herpes simplex virus (HSV) with acyclovir (ACV) resistance is a cause for concern due to the frequent use of ACV for treatment, suppressive therapy, and prophylaxis of HSV infection. Although HSV infection is prevalent among children, very little is known about the drug susceptibility of HSV circulating in this patient population.

Objective: To determine the status of ACV resistant HSV-1 among children.

Study design: A reporter cell-based HSV infection assay (mVILA) was developed to conveniently evaluate the ACV susceptibility of HSV-1 clinical strains and used to analyze 68 HSV-1 primary isolates from oral lesions in children.

Results: Compared with PRA, mVILA is easier to perform. Using mVILA, HSV-1 isolates C106, C153, and C174 were found completely resistant to ACV, with a greater than 100-fold increase in IC50s. Sequence analysis of thymidine kinase (TK) and DNA polymerase (DNA POL) genes identified 11 new mutations. Structural modeling of the TK and DNA POL proteins suggested structural changes that might alter their interactions with ACV and ACV triphosphate, respectively. The insertion of a single G in a seven-guanine homopolymeric repeat sequence generated a truncated TK protein in C106.

Conclusion: This study provides preliminary data on the ACV susceptibility status of HSV-1 in children. The prevalence rate of ACV-resistant HSV-1 in children was higher than predicted. Moreover, multiple mechanisms leading to the resistance were identified. These results suggest that new anti-herpetic drugs with different working mechanisms should be valuable.

1. Background

Acyclovir (ACV) is the first selective antiviral drug to be characterized; it has been the drug of choice for the prophylaxis and treatment of primary and recurrent HSV infection for more than two decades now. ACV is a nucleoside analog prodrug that can be converted into an ACV monophosphate by the HSV thymidine kinase (TK) within HSV-infected cells. Cellular kinases subsequently add two more phosphates to form the active drug ACV triphosphate (ACV-TP). The ACV-TP competes with 2-deoxyguanosine triphosphate as a substrate for viral DNA polymerase (DNA POL), thus terminating DNA synthesis upon its incorporation. Over the years, ACV-resistant (ACV-r) HSV mutant strains have been isolated from both immunocompromised and immunocompetent adult patients.1,2 Using standardized or non-standardized methods, the prevalence rates of ACV-r HSV were reported vary from 3.5% to 10.9% in immunocompromised patients and from 0.3% to 0.7% in immunocompetent patients.2–9 Among these ACV-r HSV mutant strains, 95% and 5% are caused by TK gene mutations and the viral DNA POL gene, respectively.7,10

Studies on ACV-r HSV strains have suggested that TK mutants produce distinctly altered TK enzymes, such as TK-negative mutants, mainly because of the disruptive characteristic of the TK ORF through deletion/insertion mutants, and TK mutants with reduced ACV sensitivity from the structural alteration of the TK protein.11 Although rare, HSV DNA POL mutants have been identified from ACV-r clinical isolates.12,13

Children are susceptible to infection by HSV-1, a common cause of ulcers in the mouth, gingivostomatitis, cold sores, or oral/labial lesions. However, the status of ACV-r HSV among children has not
been reported yet, partly due to the lack of clinically practical drug susceptibility assays.

Phenotypic and genotypic methods have been designed to determine the ACV susceptibility of HSV clinical samples. Genotypic tests were mainly performed through direct sequencing of tk and DNA pol to determine viral susceptibility based on databases of mutations related to ACV resistance. On the other hand, phenotypic evaluations, including plaque reduction assay (PRA), dye uptake assay, and virus yield reduction assay, were largely conducted on HSV infections that induced the cytopathic effect. These assays are time consuming, laborious, and hence inconvenient for routine clinical screening. We recently reported on the development of the Vero-ICP10-Luciferase (Vero-ICP10-Luc) assay (VILA) for the screening of antitherapeutics that target the early stages of HSV infection. This assay relies on a reporter-expressing cell line to quantitatively measure HSV infection.

2. Objectives

To determine HSV drug susceptibility in clinical settings, we modified VILA (mVILA) and compared with PRA. We applied mVILA to determine the ACV susceptibility of 68 HSV-1 isolates from a cohort of children in Guangzhou, China. The tk and DNA pol of the ACV-r strains were sequenced. Finally, the potential mechanisms of the ACV-r phenotype were analyzed through protein structure modeling.

3. Study design

3.1. Cells, virus, and antiviral drugs

The Vero-ICP10-Luc cell, which encodes the firefly luciferase gene under the HSV-2 ICP10 promoter, was previously constructed in our lab. Vero-ICP10-Luc cells were maintained in DMEM supplemented with G418 (300 µg/mL) (Sigma, USA). HSV-1 F (ATCC No. VR-733) and HSV-1/Blue, a TK mutant derived from HSV-1 (KOS) (from Profs. Gary H. Cohen and Roselyn J. Eisenberg, University of Pennsylvania), were propagated as previously described. ACV (Sigma) was dissolved in water and stored as 0.5 mg/mL stock at −20°C.

Sixty-eight clinical isolates of HSV-1 were collected from the oral lesions of children (3–5 years old) from outpatients in Guangzhou Children’s Hospital, Guangzhou, China. Viral isolates were obtained through tissue culturing with Vero cells, and the viruses were identified through PCR based molecular diagnostic methods and confirmed through sequencing.

3.2. mVILA and PRA for HSV ACV susceptibility analysis

mVILA was performed as follows: Vero cells were plated in a 96-well culture plate and cultured to monolayer overnight. A 100 µL aliquot of the test virus in 8 different concentrations of ACV (seven 4-fold dilutions starting from 32 µg/mL and a blank with no ACV) was inoculated into the cells in triplicate. The supernatant medium was removed after incubation at 37°C for 20–21 h, and a 100 µL volume of 3 x 105 cells/mL fresh Vero-ICP10-Luc cells was overlaid. Luminescence intensity was measured after incubation at 37°C for 7 h. The 50% inhibitory concentration (IC50) was calculated by linear regression analysis in at least two independent experiments. In each experiment, HSV-1 F was used as the wildtype ACV sensitive virus control, and HSV-1/Blue (TK null mutant) was used as the ACV resistant virus control. Relative IC50 (rIC50) between the testing isolates and HSV-1 F were calculated, and rIC50 > 3 was used as breakpoint for ACV-r strains.

For PRA, 90% confluent Vero cell monolayer in a 24-well plate was inoculated with 40–60 PFUs of virus with serially diluted ACV in DMEM. The culture medium containing different concentrations of ACV and 1% methylcellulose was added to the wells after incubation at 37°C for 1.5 h. The cells were fixed and then stained with crystal violet (Sigma).

3.3. Sequencing of TK and DNA pol genes

The TK gene was sequenced using the TK-F (5′-GGGATTCGAGATGATGAG-3′) and TK-R (5′-GGTTCCAGTTCCACTTGCAATA′) primers pairs, whereas the DNA pol gene was sequenced by the LP6 (5′-GACACGTCTCTGTTTTT-3′) RP7 (5′-CGGAGACGGTATCGTGTA′) primer pair and the overlapping primers RP6 (5′-CCAGTTACACAGACCTTG-3′) and LP7 (5′-GATTAACATCCCGCCACC-3′). All mutations were confirmed through sequencing of at least two PCR amplicons.

3.4. Mutant model building

Modeling and energy minimization for the mutations were performed using Discovery Studio (version 2.1, Accelrys, Inc.). Crystal structures of the wild type of HSV-1 TK in complex with ACV (PDB ID 2KIS24 and HSV-1 DNA pol (PDB ID 2G922523) were chosen as the templates for the corresponding mutant modeling. The structural models of the HSV-1 TK A365T mutant and the HSV-1 DNA pol P920S mutant were constructed using the Build Mutants protocol as implemented in the Discovery Studio 2.1 program suite. For direct comparison, the same successive steepest descent and conjugate gradient algorithms were applied in the energy minimization of both wild-type and mutant models. Figures were prepared using PyMOL.

4. Results and discussion

4.1. mVILA for HSV drug susceptibilities

VILA uses a reporter cell line that expresses Luc upon HSV infection. After optimization, mVILA was designed to integrate into clinical procedures to assess the level of ACV sensitivity of HSV-1 (Fig. 1). With mVILA, the IC50 of the isolated virus is calculated and compared with an ACV-sensitive HSV-1 reference strain (e.g., HSV-1 F) and an ACV-r HSV-1 reference strain (e.g., HSV-1/Blue, a TK null mutant virus).

Different PFUs of the HSV-1 F and HSV-1/Blue were assayed in the absence of ACV to determine the correlation between the Luc value and the infectious viral particle number. The value of Luc activity increased linearly with increasing PFUs over the broad range of (20–2) x 106 PFU/mL, suggesting that the value of Luc could be used to calculate the number of infectious HSV-1 in the cell culture.

PRA is the gold standard in evaluating HSV-1 drug susceptibility despite its tedious and laborious analytical approach. In addition, PRA is quantified by counting plaques, which limits its throughput and is subject to human error. In view of these limitations, PRA is currently not used in routine clinical settings for drug susceptibility analysis of clinical isolates. In this study, the ACV IC50 values of 20 HSV-1 clinical isolates were measured with mVILA and PRA in parallel to determine whether mVILA could replace PRA. The rIC50 values obtained by VILA correlated well with those by PRA for this group of viruses (r = 0.8113, P < 0.001; Fig. 2B).

Taken together, compared with PRA, mVILA is a quantitative and more convenient assay in clinical settings.
4.2. ACV susceptibility analysis of HSV-1 isolates from children

HSV-1 from oral lesions were isolated from 68 children between 3 and 5 years old in Guangzhou Children’s Hospital, Guangzhou, China, to evaluate the ACV resistance status of HSV-1 in this patient population. Of the HSV-1 isolates (Fig. 3, The values of IC50s are in Supplementary Table 1), 65 had rIC50 values below 3 and were thus considered as ACV sensitive. However, the rIC50 values for C106, C153, and C174 were higher than 100, as was the case for the recombinant TK null strain HSV-1/Blue (rIC50 = 158.8). These strains were thus classified as completely resistant to ACV.

4.3. Sequence analysis of the TK and DNA POL genes of the isolates

From other studies, approximately 95% of ACV-r HSV mutant strains involve the TK gene, whereas the remaining 5% are caused by the viral DNA pol gene. The tk of the 68 strains evaluated in the present study were sequenced to better understand the HSV-1 isolates’ mechanisms of differential sensitivity to ACV.

Twenty-one mutations were identified from these strains. Compared with the HSV-1 F tk sequence (Table 1), 11 mutations have not been previously reported. None of the mutations was found within the conserved regions (Fig. 4C, marked as black blocks). The results for the three strains completely resistant to ACV were interesting: a G insertion at position 437 within the seven-guanine nucleotide stretch at positions 430–436 was found in C106; this insertion generated a nonfunctional truncated protein. The TK sequence of C153 included V348I, L42P, and Q89R, which were also found in the ACV-sensitive strains of the present cohort, indicating that they might not yield the completely resistant phenotype. V905M and P920S were identified from its DNA POL. As V905M has been earlier reported as a polymorphism, P920S possibly contributed to the ACV-r phenotype.

The only mutation identified from the DNA POL of C174 was V905M, indicating that its DNA POL might not be the source of the ACV-r phenotype. Two mutations were identified from its TK: D14H and A365T, with the latter mutation being specific to this TK sequence.

4.4. Structural insight into the drug-resistant mutants

As C153 showed a greater than 100-fold increase in its IC50 value for ACV, the potential impact of A365T in C174 on the TK protein structure and its interaction with ACV were modeled. The co-crystal
structure of HSV-1 TK with ACV showed that Ala365 is located in the middle of α-helix 12,27 which is not directly involved in the formation of both ATP and ACV-binding sites. However, Ala365 lies opposite to β-sheet 2 (β2), followed by one loop, in which the Glu83 side chain forms one hydrogen bond to the hydroxyl group of ACV. This substitution would influence the orientation of β2 and subsequently trigger movement to the connected loop region when Ala365 is displaced by the bulkier threonine (Fig. 4A). This movement would likely change the position of Glu83, disrupting the hydrogen bond to the hydroxyl of ACV. The minimized mutant structure in the present study clearly showed that the volume of the binding cavity for ACV decreased from 930 Å³ in the wild type to 750 Å³, as calculated by VOIDOO.28 Apart from Glu83, changes in Trp88, Arg222, Tyr201, and Tyr172 also contributed to the decreased size of the binding pocket (Fig. 4B). The ACV resistance of the mutant A365T could hence be explained by the disruption of Glu83 hydrogen bonding and shrinkage of the binding pocket.

C174 had a rIC50 of 112.8 for ACV. The mutation P920S was located in a loop region whose distance from the ACV-TP made

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**Fig. 3.** The rIC50s of HSV-1 isolates. ACV IC50s were measured with mVILA as described in Section 3.2. The IC50 values are summarized in Supplementary Table 1. rIC50 represents the ratio of IC50s between isolates and HSV-1 F.

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**Table 1**
The TK mutations identified in this study.

<table>
<thead>
<tr>
<th>Amino acid mutations</th>
<th>Nucleic acid changes</th>
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<tr>
<td>C6G</td>
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<td></td>
<td>G68A</td>
<td>C104, C85, C184, C207, C122, C12, C146, C214, C87, C108, C112, C126, C156, C172, C182, C177, C205, C213, C642, C745, C76, C968, C86, C184, C181, C141, C81</td>
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<td>L42P</td>
<td>T125C</td>
<td>C153, C121, C215, C166, C106, C140, C190, C75</td>
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* The mutations not reported previously are underlined.
Fig. 4. Structure modeling of C174 TK and its ACV binding pocket. (A) Potential structural changes induced by A365T mutation. The side chains of T365 and E83 are shown in light gray. (B) Binding pocket of ACV is reduced by movement of surrounding residues. The amino acids of wild type and mutant are shown in green and magenta respectively. (C) Conserved regions of TK protein and positions of mutations identified from C174. The conserved regions are indicated as black blocks. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

direct contact with it impossible (Fig. 5). However, an α-helix containing L721 and Y722, which participate in ACV-TP incorporation and provide a “steric gating” effect, was identified close to this loop. When proline is substituted by serine, the dihedral angle restriction inherent to proline would be greatly reduced, potentially causing conformational change in the loop region. A change in the 920 loop could induce movement of the α-helix containing L721 and Y722 and then influence ACV-TP binding. As a single P920S

Fig. 5. Structure modeling of C153 DNA POL. (A) Ribbon diagram of HSV DNA POL with M905, S920 and residues involved in ACV-TP incorporation (L721 and Y722) shown in stick and colored green. (B) Conserved regions of TK protein and positions of mutations identified from C153. The conserved regions are indicated as black blocks. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
substitution of DNA pol is not sufficient for ACV resistance, it was interesting to find that additional mutations, including V905M in the present study and the previously reported A645T, D671N, and A719M mutations, always displayed ACV resistance along with P920S. It is speculated that multiple mutations have an accumulated effect that could change the positions of L721 and Y722.

In summary, HSV-1 clinical strains from a cohort of immunocompetent children in southern China were investigated for their ACV sensitivity. Three strains were completely resistant to ACV, accounting for ~4% of all strains evaluated, which is approximately 10-fold higher than the rate found in immunocompromised adults but within the range determined for immunocompromised patients. Given this unexpected high ACV resistance rate in children, with the clinical more friendly mVIRA available, larger multi-centered clinical studies are required to better depict ACV susceptibility among the HSV-1 strains circulating in this patient population, and to predict the future trends of drug resistant HSV-1 in the general population. Nevertheless, the emerging drug resistance of HSV-1 requires more detailed mechanistic studies, and the potential impact for future antitherpetic development strategies should be evaluated.

Conflict of interests

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jcv.2011.06.009.

References